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14. ABSTRACT (200 words) This is our final report for the project: P11, A Biomarker for Memory Retrieval: A Possible Role in Traumatic Stress. We carried out all of the experiments based on the proposed research design. We tested memory retrieval performance with wild type and p11 knockout mice, which developed and bred by us with since JAX. There were two sets of results: control data and experimental data sets. The controls were wild-type and p11 knockout mice, and pharmacological controls (i.e., mice received saline injection) and stress controls (yoked foot shock). The experimental groups were wild-type or p11 knockout mice, which received pharmacological treatment (corticosterone injection) and foot shock exposure. We found that the latency to find platform was shorter in p11 knockout mice than that in wild type mice during training day 1-3, although the latency for both wild and knock-out was not different on the final day of training or probe test. We also found that corticosterone resulted in significant decreases in the time spent in quadrant and number of island crossing in both p11 knockout and wild type control. Our data indicate that p11 knock-out enhances memory retrieval and corticosterone induced-impairment of memory retrieval is independent of the p11 expression.					
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INTRODUCTION

Subject: Pre-clinical and clinical studies have shown the important role of p11 in Post-Traumatic Stress Disorder (PTSD) (Zhang 2008, 2009. CSTS, News from the center, 2008; CSTS, 2008 annual report; Army Annual report 2009 and Psychiatric News Oct 2, 2009). P11, also known as S100A10, is a protein encoded by the S100A10 gene in humans and is a member of the S100 family of proteins containing two EF-hand calcium-binding motifs, which are localized in the cytoplasm and/or nucleus in a wide range of cells (Santamaria-Kisiel et al., 2006). P11 is associated with both depression (Svenningsson et al., 2006) and PTSD (Zhang et al., 2008; Su et al., 2009). The evidence from animal studies supports the view that stress influences learning and memory (de Quervain et al., 1998) and increases p11 expression in the brain (Zhang et al., 2008). The most common characteristic of PTSD is the re-experiencing syndrome, when the patient's memory seems to be fixed on a traumatic event whereas the processing of non-trauma-related memories is often impaired. Therefore, these results led us to hypothesize that p11 might play an important role in memory. It is found that footshock given 30 minutes before memory testing impaired rats' retention performance in a water-maze spatial task, compared to non-stressed controls. This impaired retention performance corresponded to the levels of circulating corticosterone at the time of retention testing. In addition, the stress-induced retention impairment was blocked by metyrapone, a synthetic inhibitor of corticosterone. Furthermore, systemic corticosterone administered to non-stressed rats 30 min before retention testing induced dose-dependent retention impairment (de Quervain et al., 1998). Finally, glucocorticoid increases p11 expression *in vitro* through glucocorticoid response element sites in the p11 gene promoter (Zhang et al., 2008). Therefore, we will use wild type and p11 knock-out mice in this study to elucidate the possible role of p11, which can be regulated by glucocorticoid, in memory retrieval to facilitate the development of a therapeutic intervention for stress related disorder PTSD.

Purpose: Our immediate objective is to investigate the role of p11 in memory using p11 knockout stressed animal model, which is developed in our laboratory with The Jackson Laboratory. Our study provides an opportunity to determine the possible mechanism of stress-induced changes of retrieval memory, which are mediated by p11 and glucocorticoids. This information may translate into knowledge that will help to develop medicines for PTSD treatment. From a scientific perspective, military personnel who are exposed to trauma at higher-than-average frequencies need to have an efficient medicine to help minimize PTSD. The evaluation for the effect of p11 regulated by glucocorticoid on memory may provide an alternative and/or adjunctive therapy for PTSD seen in military psychiatry.

Scope:

- Innovation: This study will provide information about the molecular mechanism of underlying memory and p11 in stress and fill a knowledge gap in the current research on PTSD. Such knowledge may facilitate the development of novel pharmacological interventions for the treatment of PTSD.
- Intervention: Administration of glucocorticoid related agents may help to prevent as well as treat PTSD. More specific interventions, targeting p11 gene provide treatment while minimizing side effects.
- Application: Interventions of p11 gene expression have the potential for use both in military populations (those on active duty, reservists and veterans) and in civilian populations exposed to traumatic stress (natural disasters, vehicle crashes, etc.).

BODY

Task 1: Animal protocol approvals and P11 knockout mice model

Announcement of Concept Award W81XWH-08-2-0202 to Lei Zhang 2008. The Certificate of Environmental Compliance for this project was awarded by the USUHS Environmental Compliance Officer on January 15, 2008 and the PI Assurance Document was signed January 15, 2008. Our animal protocol was approved on September 29, 2008. A copy of the IACUC approved animal protocol was submitted to the USAMRMC ACURO for review and received ACURO approval. Since this experiment is using p11 knockout mice and the mouse is not valuable in the market at that time, we had to develop a p11 knockout animal protocol our own.

To develop a p11 knockout provides a great opportunity to study the possible role of p11 in memory and in PTSD. However it is also a challenge, scientifically and administratively. Originally, we were planning to develop p11 knock-out mice at Children's National Medical Center in Washington, DC, considering their low cost, proximity, and expertise. Unfortunately, they were changing personnel who were responsible for breeding our p11 knock-out mice in the hospital, so we had to search for a new knock-out facility. We had searched several possible companies, but the cost for development p11 knock-out mice was much higher than we had proposed. Fortunately, through long negotiations and discussions of the technical issue on the development of p11 knock-out mice, we found that the Jackson Laboratory could develop p11 knockout mice with our proposed cost.

The Jackson Laboratory is an independent, nonprofit organization focusing on mammalian genetics research to advance human health. Their mission is to discover the genetic basis for preventing, treating and curing human disease, and to enable research for the global biomedical community. They have more than 1,400 employees located in Bar Harbor, Maine, and in Sacramento, California and conduct genetic research, providing scientific services and genetic resources to laboratories around the world. They have used wild type and knock-out mice as research tools. The mouse genome is 95 percent identical to the human genome and is therefore an effective and efficient model for human diseases. Jackson Laboratory is the major provider of knock-out mice in US. Since developing p11 knockout mice is an innovation, there is intellectual (material transfer) property issue. It needs to have a material transfer agreement from all this research project related institution. We were able to order the p11 knockout mice from The Jackson Lab with an agreement signed by the institutions, including MRC Harwell, our university and Jackson Lab. We ordered specific frozen mice embryos from MRC Harwell and created a p11 knockout colony at The Jackson Lab. The p11 knockout mice were breeding and genotyped at Jackson Lab. Our university negotiated with Jackson Lab since August 9, 2010. The final agreement was reached by all side on March 9, 2011. Thus, we first conducted experiments using wild type. Then we did the experiment with our p11 knockout mice. Fortunately, with your kindly supporting, we were able to extend our project and obtained the p11 knockout mice from Jackson to conduct the experiments with p11 knockout mice. All animal experiments have been done in accordance with IACUC and ACURO approved protocols.

The p11 knockout mice are one of important tools to deeply investigate p11 gene function. We developed p11 knockout mice colony using a conditional knock-out approaches to accomplish our Task one. Thus, p11 knockout creates a model which mimics human conditions to study PTSD, stress, particularly the memory. We conducted the following experimental approaches to obtain p11 knockout mice (conditional S100A10-null mice): Exon 2 of p11 is flanked by loxP sites and the first translated region of the S100A10 gene. A 200 base pair probe for p11 was prepared by PCR, using 129-derived embryonic stem cell DNA as a template with primers (5'-gccaaactggaggcactggtacccccc-3' and 5'-ggatacaacaataaaaaactcagaagc-3'). This probe was used to identify genomic clones from an RCPI-22 129S6/SvEvTac mouse bacterial artificial chromosome (BAC) library. The targeting vector was derived from two overlapping BamHI and ApaI genomic sub-clones that contained both exons 2 and 3 of p11. The 5' arm is the 4412 bp BamHI-XbaI fragment upstream of exon 2. The loxP-flanked (floxed) exon 2 was obtained as a 990 bp XbaI-ApaI fragment. The 3' arm is the 6422 ApaI-XmaI fragment downstream of exon 2. The three genomic fragments were inserted into a plasmid containing two loxP sites and an FLP recombination target-flanked [FRT-flanked (flrted)] neo cassette. The complete targeting vector was linearized and electroporated into 129-derived embryonic stem cells and screened, using 5' and 3' external probes for Southern blot. Two correctly targeted clones were injected into C57BL/6J blastocysts to generate chimeras. These chimeras were crossed to C57BL/6 animals. This was followed by a cross to FLP deleter mice to excise the positive selection marker. Correct targeting was confirmed by Southern blot. The numbers of knockout mouse are showed in Table 1.

Secondly we conducted behavioral phenotyping when we were breeding the mice. Due to pleiotropic effects, one gene may have different functions in different organ systems or time points during development. Therefore, p11 knockout have to be phenotyped to enable the detection of phenotypes which might otherwise remain hidden. Particularly we observe the behavioral phenotype. Animal behavior can be viewed as the outward manifestation of an orchestrated and complex functioning of the central nervous system and of its interactions with the internal and external environment. In the context of functional mouse genetics, behavioral phenotype methods are applied to detect CNS dysfunctions that are relevant to human neuropsychiatric

disorders such as post-traumatic stress disorder, and other anxiety disorders. Several factors can influence behavioral phenotype results, and therefore need to be considered in the context of data reproducibility. Due to developmental and degenerative processes the age of the subject can play a role, as well as the time point of testing due to the circadian rhythms affecting many biological processes. Therefore experimental subjects and controls of the same age should be tested concurrently; we documented the phenotype and recorded the examined date for each animal. These data was used in our baseline data analysis. That was an important step for us to finish our Task 2 and Task 3.

Table. 1. Breeders and Litters

Strain	Description	Gender	Age weeks	Genotype	Quantity
005359	B6.Cg-Tg(Camk2a-cre)T29-1St/J	Breeder M	10	HOM	2
005359	B6.Cg-Tg(Camk2a-cre)T29-1St/J	Breeder M	11	HOM	3
005359	B6.Cg-Tg(Camk2a-cre)T29-1St/J	Breeder M	13	HOM	5
908983	SA100A10	Breeder F	06	HOM	12
908983	SA100A10	Breeder F	09	HOM	6
908983	SA100A10	Breeder F	10	HOM	2
908983	SA100A10	Breeder F	13	HOM	2
908983	SA100A10	Breeder F	14	HOM	4
908983	SA100A10	Breeder F	15	HOM	2
908983	SA100A10	Breeder F	18	HOM	2
908983	SA100A10	Breeder F	25	HOM	3
908983	SA100A10	Breeder F	28	HOM	2
908983	SA100A10	Breeder M	13	HOM	1
908983	SA100A10	Breeder M	14	HOM	2
908983	SA100A10	Breeder M	15	HOM	1
908983	SA100A10	Breeder M	18	HOM	1
908983	SA100A10	Breeder M	25	HOM	2
908983	SA100A10	Breeder M	28	HOM	2
908983	SA100A10	Pup U	00	HOM	6
908983	SA100A10	Pup U	01	HOM	20
908983	SA100A10	Pup U	02	HOM	16
908983	SA100A10	Wean F	03	HOM	3
908983	SA100A10	Wean F	04	HOM	9
908983	SA100A10	Wean F	05	HOM	7
908983	SA100A10	Wean F	12	HOM	3
908983	SA100A10	Wean F	13	HOM	2
908983	SA100A10	Wean M	03	HOM	3
908983	SA100A10	Wean M	04	HOM	4
908983	SA100A10	Wean M	05	HOM	4

Task 2. To determine the effects of pretest administration of footshock stress or systemic corticosterone (the major glucocorticoid) on memory retrieval performance. We have examined the effects of pretest administration of footshock stress and systemic corticosterone (the major glucocorticoid) on memory retrieval performance and p11 expression in the mouse brain in p11 wild type mice. In this experiment, after footshock or treatment with corticosterone, memory performance was examined using a spatial water-maze procedure, then p11 expression levels in the hippocampus, amygdala and cortex of the mouse were determined by Western blot and real-time PCR. In this experiment, after a footshock or treatment with corticosterone, memory performance was examined using a spatial water-maze procedure. Then, p11 expression levels in the hippocampus, amygdala and cortex of the mouse were determined by Western blot and real-time PCR. All mice were housed in group cages (2-3) with free access to food and water. They were kept in the air-conditioned animal facility with a 12 hr light/dark cycle. All mice were identified by cage card. Body weight was taken before and after experiments.

We examined whether footshock or corticosterone impairs memory retrieval and alter the expression of p11 in discrete regions of the mouse brain. If stress-induced memory retrieval impairment does not alter p11 expression, such findings would indicate that stress-induced retrieval impairment is independent of glucocorticoid-regulated P11 expression.

In the memory test, the animals were placed into the water at and facing the sidewalls of the pool, at different start positions across trials, where they quickly learn to swim to the correct location with decreasing escape latencies by more direct swim paths. The tracking system measures the gradually declining escape latency across trials, and parameters such as path-length, swim-speed, and directionality in relation to platform location. Mice received one training session each day for 10 days. Each daily session began with a single reinforced probe trial, followed by four training trials. For the probe trials, the platform was lowered so that it was inaccessible and the mouse were placed in the water facing the pool wall at one of four start points (north, south, east, or west). The start points were counterbalanced across trials for all animals. Upon release into the water, the mouse was allowed to swim for 60 sec, at which point the platform was raised to within 1.5 cm of the water surface. An additional 60 sec were then allowed for the mouse to locate the platform and escape from the water. After escaping, the mouse remained on the platform for 30 sec before being removed. If the mouse fails to escape, it was guided to the platform and remained there for 30 sec. After completion of the daily probe trial, four training trials were given with the platform in the raised position (1.5 cm below the water surface) so that it will provide a means of escape from the water. The procedure was the same as for the probe trials, except that the mouse was allowed 120 sec to find the platform. On completion of training, mice were assigned to an immediate stress or non-stressed group. Stressed mice were stressed with food shock. Non-stressed mice were used as controls. We conducted a probe trial in which the escape platform was removed from the pool and each animal allowed to swim for 60 sec. A well-trained mouse swam to the target quadrant of the pool and repeatedly across the former location of the platform until starting to search elsewhere. This spatial bias will be used to constitute evidence for spatial memory. We found that footshock (30 min) resulted a significant decrease in the time spent on target, while producing no effect on time spent on opposite, indicating that stressed mice had significant impaired performance in the water-maze spatial task compared to control in p11 wild type (Fig 1). The procedures using animals have been approved by IACUC of our university. Briefly, a mouse was placed in a dark shock chamber and is acclimatized for 5 Min. The mouse was then subjected to shock trials from which it cannot escape. Each shock (the unconditioned stimulus) is 0.45mA, lasts 15 seconds.

Fig 1.

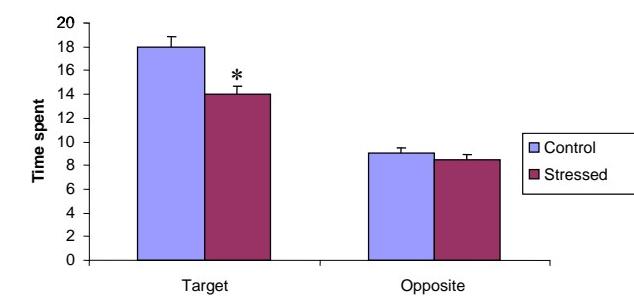


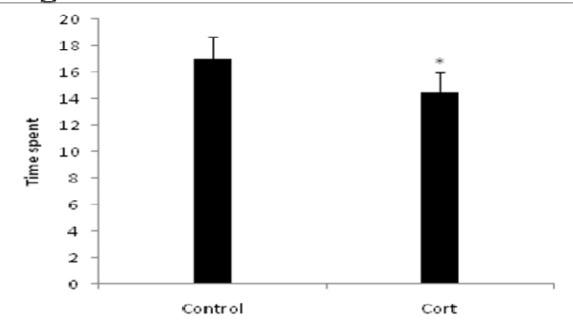
Fig 1. The effect of stress on memory retrieval. Mice had impaired performance in the water-maze spatial task after being given footshock 30 min compared to control in p11 wild type mice. P < 0.05

In this experiment, we also carried out the water-maze spatial task performance of animals treated with saline and animals treated with corticosterone and found corticosterone treatment resulted significant decrease of time spent, indicating a glucocorticoid-induced impaired memory retrieval performance (Fig 2).

Fig 2. Effect of corticosterone one retention impairment. Cort, corticosterone. *p < 0.05

To conduct Western blot, we tested p11 antibodies: In this study we were able to validate the p11 antibodies used in Western blot experiments. Those antibodies were purchased from several companies, including R and D System, Novus Biologicals, Bio Red and Antibody Verify. Since western blotting remains one of the most common scientific methods for monitoring protein expression in cells or tissues, we have tested those antibodies by western blot using mice tissues. The accuracy of western blot results relies heavily of the quality of the primary antibody employed in the immunoblotting. Currently, most p11

Fig 2.



antibodies have been developed for protein assay in human and rats. There are few antibodies against p11, which have been tested in mice. Therefore, we tested the specificity and sensitivity of p11 antibody. Specifically, we have done the antibody validation experiments in the brain of mice. We also examined p11 expression in the animal brain using an immunohistochemistry.

Table 2. The Western blot testing antibodies in mice brain

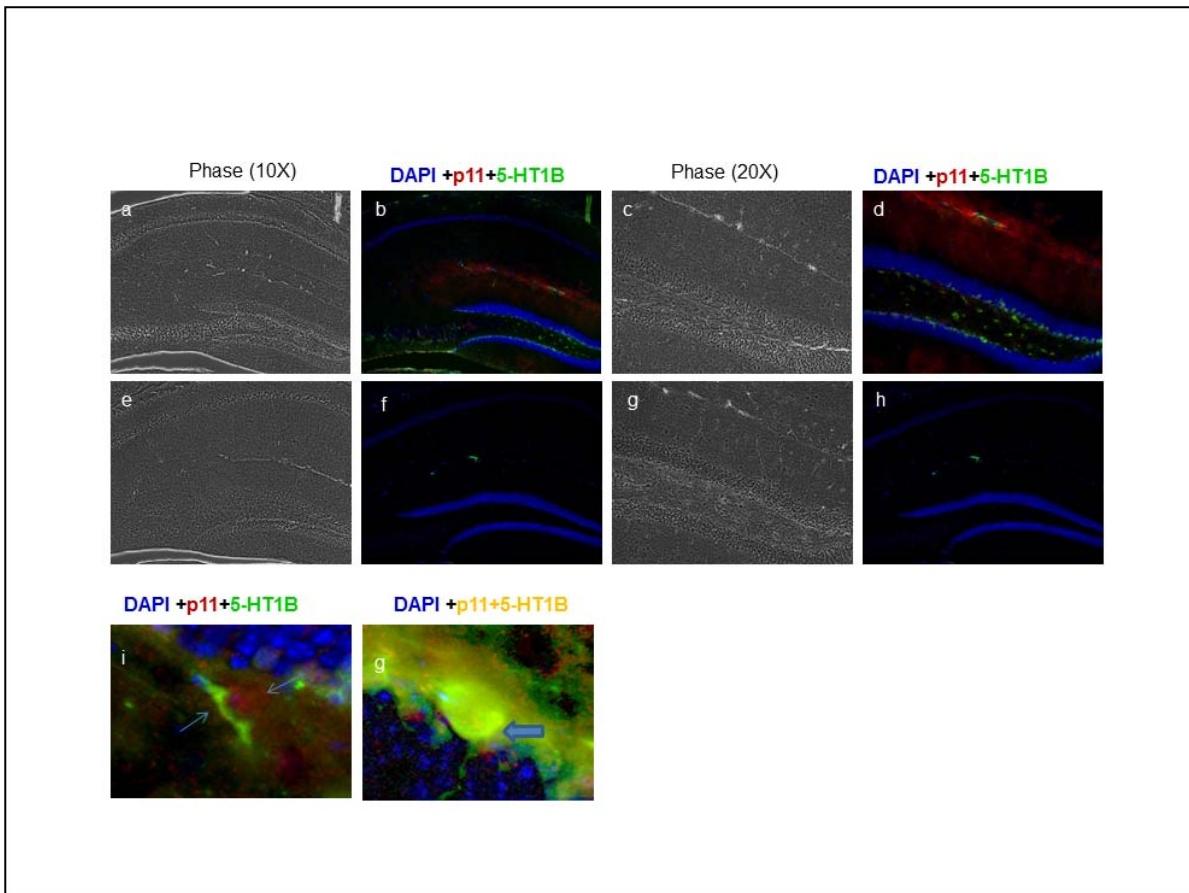
Antibodies	Immunogen	Immunoactivity	Species Reactivity
Mouse S100A10 Affinity Purified Polyclonal Ab, Goat IgG	E. coli-derived recombinant mouse S100A10. Pro2-Lys97 Accession Number P08207	Western Blot (+)	Mouse, S100A10 in Western blots.
S100A10 Antibody Cat# AAS9363C	Rabbit, EFPGFLENQKDPLAVD KIMKLDQCRDGKVG FQSFFSLIAGLTIACND YFVVMKQKGKK;	Western Blot (+)	Cross-reactivity with p11 in Bovine; Human; Rat; Dog; Horse; Mouse
S100A10 Antibody NBP1-19756	Mouse Monoclonal	Western Blot (-)	Cross-reactivity with p11 in Human, Rat
S-100 β chain Antibody (C-20)	A goat polyclonal IgG	Western Blot (+)	Cross-reactivity with p11 in mouse, rat and human

Each individual p11 antibody is capable of binding specifically with one unique epitope. This specificity allows precise detection of p11 antigen as a protein while avoiding detection of unrelated proteins. However, it is important to recognize that a particular p11 epitope could potentially appear on more than one protein antigen. Therefore, one p11 antibody could potentially recognize two or more proteins if these proteins are highly homologous and contain the same epitope. In addition, multiple antibodies have been generated against p11 protein antigen. Any one of these antibodies may potentially cross-react with another protein that contains the same epitope(s). There is also the issue of how p11 antibodies are typically used, since they can be collected directly from the serum or by isolating the B cell(s) that is producing the antibody of interest. These two antibody production techniques relate to antibodies: polyclonal and monoclonal. In general, p11 antibody specificity is important in our study. We must determine which antibody is used in our research (polyclonal and monoclonal), and which is able to detect our target p11 protein specifically without cross-reacting with non-specific proteins in mice.

First, we tested the p11 polyclonal antibodies, which represent the antibodies from different B cells that recognize multiple epitopes on the same p11. Each of these individual antibodies recognizes a unique epitope that is located on p11. They might be higher overall antibodies affinity against the p11 due to recognition of multiple epitopes. In general, ability to detect multiple epitopes gives more robust detection. They should offer greater sensitivity for detecting p11 that is present in low quantities in our sample since multiple antibodies will bind to multiple epitopes on the protein. The disadvantages are variability between different batches produced in different animals at different times, and higher potential for cross reactivity due to recognizing multiple epitopes.

Then, we also tested the p11 monoclonal antibodies in our study. P11 monoclonal antibodies, by contrast, represent antibody from a single antibody producing B cell and therefore only binds with one unique epitope. Their advantages are: batch to batch homogeneity, high specificity to a single epitope and reduced probability of cross reactivity. Their disadvantages are: more expensive, small changes in the epitope's structure render the monoclonal antibody unable to detect the p11 protein and less robust for detecting the p11 in a denatured state or altered conformation, less ideal for application requiring quick capture of the p11 protein, and more sensitive to pH and buffer conditions. To offset many of these disadvantages, it is necessary to produce a pool of several monoclonal antibodies. This is typically costly and time prohibitive. We selected one of the

antibodies (Mouse S100A10 Affinity Purified Polyclonal Ab, Goat IgG), which is commercially available for immunohistochemistry validation experiments.



Verification of labeling of p11 positive neurons in p11 knockout and wild type mice by immunohistochemistry: In validation of immunohistochemistry studies, mice brains were fixed by vascular perfusion of 2% paraformaldehyde in PBS (pH 7.4). The brains were cut into 20- μ m sections with a Vibratome (The Vibratome; St. Louis, MO). Tissue sections were mounted in anti-fading mounting medium (Vectashield; Vector Laboratories) on glass microscope slides and examined with a Zeiss Axiohot fluorescence microscope. Above Fig shows immunocytochemistry of hippocampal neurons by using antibodies anti-p11 (S100A10 protein), and 5-HT1B receptor antibody as well as DAPI. ZEISS micrographs of neurons from wild type (a, b, c, d) labeled with DAPI and p11 antibody (a, b) and (wild type, c, d) double-labeled for p11 (red) and 5-HT1B (green). P11 immunoreactivity was prevalent at the hippocampal neurons of wild type mice (a, b, c, d) but negligible in the neurons of p11 knockout mice (e, f, g, h $-/-$) mice. Double labeled neurons in wild type was also evidenced (i, g arrow).

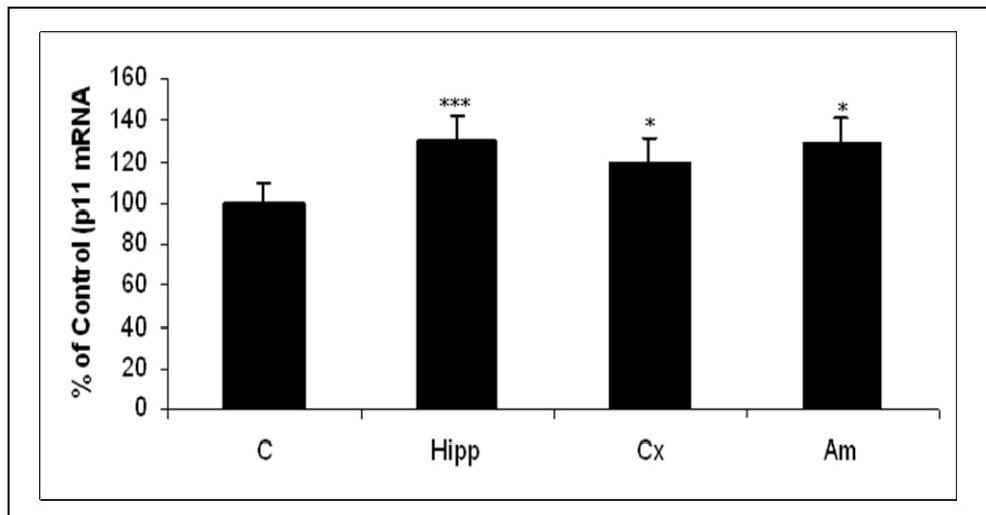
Colocalization of labeling using two antibodies directed against p11 and 5-HT1B receptor in the mouse hippocampus. Double-labeling using the two antibodies (polyclonal anti p11 and monoclonal anti-5-HT1B) revealed that the two antibodies can label the same neurons in mice hippocampus, the p11 (red) labeled most strongly in the cytoplasm and nucleus, which also labeled with DAPI (blue), while the 5-HT 1B receptor antibody (green) labeled most strongly in the plasma membrane.

Summary of verification of p11 antibody by immunohistochemistry in mice brain: There were p11 positive neurons in wild type mice, but not in p11 knockout. P11 is specifically expressed in the neuronal cells expressing 5-HT1B receptor, indicating the role of p11 in the regulation of 5-HT1B receptor in the hippocampus of mice brain.

Task 3. To determine the effects of pretest administration of footshock stress on p11 expression in the mouse brain by Western Blot. The report for this task is the same as last report: We also examined the effect of stress on p11 expression in the hippocampus and amygdala by Western blot. The procedure for this experiment follows in the proposed methods. Briefly, control (n=10) and stressed mice (n=10) were decapitated. The brain was excised from each and sliced coronally using a vibratome. The hippocampus and amygdala were dissected from the slices and placed on dry ice immediately for Western blot and p11 mRNA expression studies. Protein concentration in the samples was determined by Bio-Rad Protein Concentration Reagent (Hercules, CA). Equal amounts of total protein (20 µg per lane) were resolved in 10% SDS polyacrylamide gels and blotted onto PVDF membranes for immunoblotting analysis. Protein expression was detected using a 1:500 dilution of mouse anti-p11 monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ) with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG as a secondary antibody (Bio-Rad Laboratories, Hercules, CA). The density values are presented as means \pm S.D. from three experiments. The density was used to quantify immunoreactivity in terms of percentage of p11 induction relative to control (non-stressed mice).

Using Western blot, we found that stress results in increases of p11 expression in the three brain regions, indicating that p11 was up-regulated by stress exposure in wild type mice (Fig3).

Fig 3. Stress resulted in p11 up-regulation, as determined by Western Blot in the hippocampus and amygdala of wild type mice. The data were analyzed by Student's *t*-test; * P<0.05, *** P<0.001. C, Control; Hipp, Hippocampus; Cx, Cortex; Am, Amygdala.



Task 4. To determine p11 mRNA expression in the

mouse brain by real-time PCR. In this experiment, after footshock or treatment with corticosterone, memory performance will be examined using a spatial water-maze procedure, then p11 mRNA expression levels in the hippocampus, amygdala and cortex of the mouse will be determined by real-time PCR. In our lab brain tissue is placed in a 10 ml Wheaton® glass homogenization tube, which increases the entire volume (tissue + buffer) to a total of 2 ml with the homogenization buffer. An appropriate sized pestle is placed in the homogenization tube containing the organ and buffer. Brain tissue is then homogenized using the tissue grinder: RW16 Basic S1 Overhead stirrer (IKA® works Inc., Wilmington NC) set at a speed of approximately 1300 rpm. Going up and down the tube only twice, the pestle keeps each sample homogenous. Based on the volume, homogenate is poured into a 2 ml tube and sonicated for 30 seconds with the VirSonic sonicator.

We use organic extraction protocols to begin the lysis and homogenization of brain tissues in a proprietary, monophasic solution of phenol and guanidinium isothiocyanate. Chloroform is then added, followed by centrifugation to separate the RNA from gDNA and proteins. The aqueous phase is removed and the RNA is precipitated, washed, and solubilized in RNase-free water. Using organic extraction, this procedure yields RNA of the highest quality.

At the end of the extraction procedure, quantitation is done by taking the spectrophotometric absorbance at 260 nm (Eppendorf Biophotometer). Assessment of RNA quality is done by both electrophoresis and the calculation of a spectrophotometric A260/A280 ratio. The A260/A280 ratio falls in the range of 1.8 - 2.2. Electrophoresis is performed on an agarose gel for samples in which 1µg can be spared for the analysis. Electrophoresis results show two strong, distinct bands representing ribosomal RNA and a light smear behind the ribosomal bands

representing messenger RNA. In addition, significantly higher molecular weight products indicative of contaminated genomic DNA are not observed.

RNA was extracted from mouse hippocampus and amygdala tissue lysates using TRIzol. cDNA was generated from 3ug of total RNA using Superscript III RT (reverse transcriptase) and oligo (dT) primers (Invitrogen). Real-time PCR was performed on the generated cDNA product in the IQ5 system using SYBR Green (Bio-Rad). The following sequences for p11 mRNA analyses were used: forward 5'-TGCTCATGGAAAGGGAGTTC-3' and reverse 5'-CCCGCCACTAGTGATAGAA-3' primers. Beta-actin mRNA levels were unchanged by treatment and were used as an internal control for normalizing p11 mRNA levels in control and experimental samples. Sequences for beta-actin primers were as described by Applied Biosystems. Dilution curves confirmed the linear dependence of the threshold cycle number on the concentration of template RNAs. Relative quantification of p11 mRNA in control and experimental samples was obtained using the standard curve method. Statistics were performed using GraphPad Prism (GraphPad Software, Inc. San Diego, CA).

We found stress and treatment of corticosterone resulted in increases of p11 mRNA expression levels in the hippocampus of wild type mice. Our results suggested stress up-regulated p11 at gene levels in the hippocampus, a brain region associated with memory (Fig4).

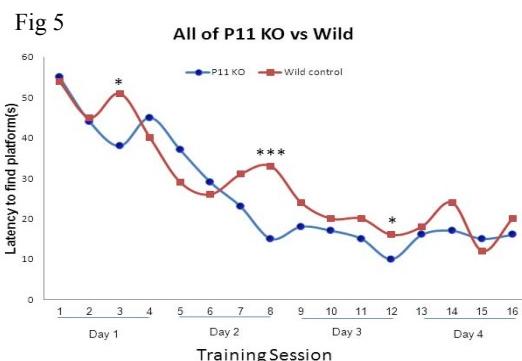
Fig. 4. The effects of stress and corticosterone on the p11 mRNA levels in mice hippocampus. Both stress and corticosterone (Cort) increased p11 expression levels in wild type mice hippocampus. The data were analyzed by Student's *t*-test; P < 0.05

Summary of Experimental Results

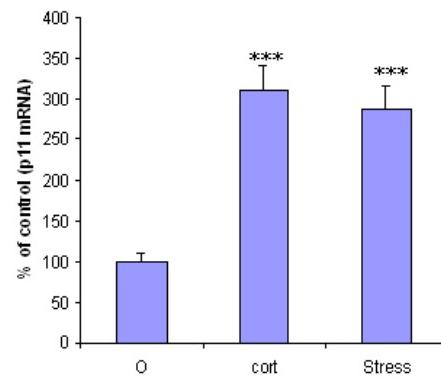
- Footshock and corticosterone significantly decreased the time spent on target and produced no effect on time spent on opposite, indicating a significant impaired performance in the water-maze spatial task in stressed mice compared to control in p11 wild type.
- Stress resulted in p11 protein up-regulation, as determined by Western Blot in the hippocampus, cortex and amygdala of wild type mice.
- Stress and corticosterone resulted in p11 mRNA up-regulation, as determined by real-time PCR in the hippocampus, a brain region associated with memory function.

Task 5. Testing knockout mice: In this study, we examined p11 knock-out mice in the water-maze spatial test to elucidate the role of p11 in memory retrieval. There were two sets of results: control data and experimental data sets. The controls were wild-type and p11 knockout mice, and pharmacological controls (i.e., mice received saline injection) and stress controls (yoked foot shock). The experimental groups were wild-type or p11 knockout mice, which received pharmacological treatment (corticosterone injection) and foot shock exposure.

We found that the latency to find platform was shorter in p11 knockout mice than that in wild type mice during training day 1-3, although the latency for both wild and knock-out was not different on the final day of training



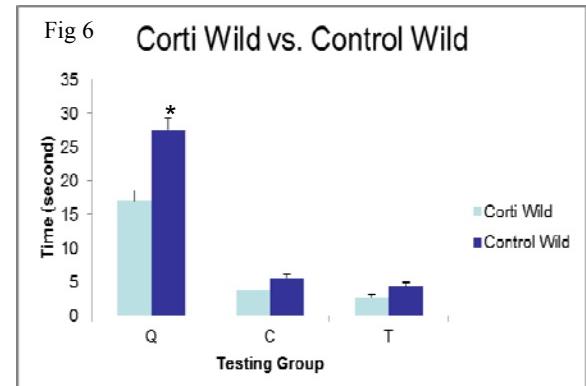
Corticosterone- and stress-increased p11 mRNA in hippocampus



or probe test (Fig 5). We also found that corticosterone resulted in significant decreases in the time spent in quadrant and number of island crossing in both wild type control (Fig 6) and p11 knockout (Fig 7). Our data indicate that p11 knock-out enhances memory retrieval and corticosterone induced-impairment of memory retrieval is independent of the p11 expression.

KEY RESEARCH ACCOMPLISHMENTS

- To get knockout mice and footshock protocol approval at USUHS.
- Design and negotiate with companies to develop our own p11 knockout mice.
- Examine the effect of footshock and corticosterone on memory preference in wild type mice using proposed protocol.
- Examine the effect of footshock on p11 protein expression in the three brain regions which are associated with memory using Western Blot.
- P11 antibodies and Western Blot procedure were tested and validated in wild type mice.
- Examine the effect of footshock and corticosterone on p11 mRNA expression in hippocampus, which plays a major role in memory retrieval.
- Real time PCR procedure were tested and validated in wild type mice.
- Breeding and genotyping of P11 knockout mice.



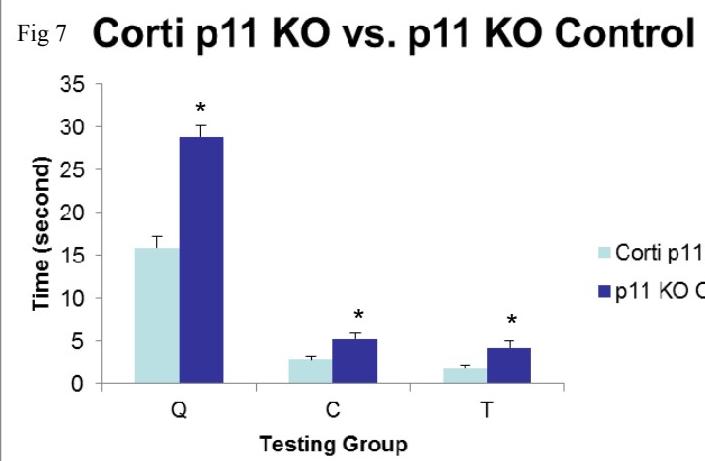
REPORTABLE OUTCOMES

- Oral and poster presentation at the Military Health Research Forum, August 31-September 3, 2009 in Kansas City, MO.
- Poster: P11, a biomarker for memory retrieval: a possible role in traumatic stress. USUHS Research day 2013.
- Poster: Extinction of Fear. The Amygdala Conference, USUHS, Bethesda, MD 2013

CONCLUSION

Our results indicate that besides the well described effects of stress and glucocorticoids on the acquisition and consolidation processes, stress and glucocorticoids also affect memory retrieval mechanisms, p11 protein expression in the hippocampus, cortex and amygdala and p11 mRNA expression in the hippocampus in wild type mice. Stress and glucocorticoids resulted in p11 over expression in the mouse brain. The role of p11 in memory retrieval was determined in p11 knock-out mice.

In general, our findings seem to provide additional evidence of p11 in PTSD and traumatic stress. We found the latency to find platform of p11 knockout mice was shorter than non-p11 knockout (wild type) mice during the training day 1-3, although both their latency was the same on the final day of training or probe test. These data indicate that p11 delete in the mice might enhance learning. Corticosterone results in significant decreases in the time in quadrant and number of island crossing in both p11 knockout and wild type control, suggesting that corticosterone induced impairment of memory retrieval, which independent the p11 expression. This information may translate into knowledge that will help to develop an alternative and/or adjunctive therapy for PTSD.



REFERENCES

Christine Creenan-Jones. Army doctor seeks novel approach for diagnosing PTSD. Military Medical News, May 31, 2012 <http://www.militarymedical.com>

News from the center: http://www.centerforthestudyoftraumaticstress.org/resources/newsarticle_6-potential_biomarker PTSD_p11

Levin, Aaron. Psychiatric News: Clinical & Research News, Biomarker May Differentiate PTSD From Other Disorders, Oct 2, 2009, <http://pn.psychiatryonline.org/cgi/content/short/44/19/25?rss=1>

CTST Annual report 2008, <http://www.usuhs.mil/csts/CSTS2008AnnualReport.pdf>

Committee on Opportunities in Neuroscience for Future Army Applications; National Research Council. OCR for page 21, http://www.nap.edu/catalog.php?record_id=12500#

de Quervain DJ, Roozendaal B, McGaugh JL. Stress and glucocorticoids impair retrieval of long-term spatial memory. *Nature*, 1998; 394: 787-90.

Lindauer RJ, Olff M, van Meijel EP, Carlier IV, Gersons BP. Cortisol, learning, memory, and attention in relation to smaller hippocampal volume in police officers with posttraumatic stress disorder. *Biological psychiatry*, 2006; 59: 171-7.

Su TP, Zhang L, Chung MY, Chen YS, Bi YM, Chou YH, Barker JL, Barrett JE, Maric D, Li XX, Li H, Webster MJ, Benedek D, Carlton JR, Ursano R. Levels of the potential biomarker p11 in peripheral blood cells distinguish patients with PTSD from those with other major psychiatric disorders. *Journal of psychiatric research*, 2009; 43: 1078-85.

Yehuda R, Kahana B, Binder-Brynes K, Southwick SM, Mason JW, Giller EL. Low urinary cortisol excretion in Holocaust survivors with posttraumatic stress disorder. *The American journal of psychiatry*, 1995; 152: 982-6.

Zhang L, Li H, Su TP, Barker JL, Maric D, Fullerton CS, Webster MJ, Hough CJ, Li XX, Ursano R. p11 is up-regulated in the forebrain of stressed rats by glucocorticoid acting via two specific glucocorticoid response elements in the p11 promoter. *Neuroscience*, 2008; 153: 1126-34.